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13. ABSTRACT *(Maximum 200 words)*

I have identified A *Drosophila* gene, *sulfateless* (*sfl*) which encodes the homologue of vertebrate heparan/haparin sulfate N-deacetylase/N-sulfotransferase. This enzyme is essential for the modification and the biological activity of heparan sulfate proteoglycans (HSPG). I find that in the absence of both maternal and zygotic activities of this gene, mutant embryos develop with segment polarity phenotypes reminiscent to loss of either Wingless (Wg) or Hedgehog signaling. To analyze the function of Sfl enzymes in a specific cell-cell interaction process, I have focused my analysis on the requirement for these enzymes during Wg signaling. I report that *sfl* mutation impair Wg signaling during both embryonic and imaginal discs development, suggesting that HSPGs contribute to the reception of Wg. Sfl acts non cell-autonomously in Wg signaling and overexpression of Wg can bypass the requirement for this enzyme suggesting that HSPGs modulate Wg signaling, possibly by facilitating the binding of Wg to its receptor. These findings thus provide strong genetic evidence that HSPG is critical for signaling by Wg.

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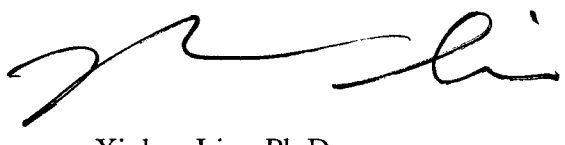

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INTRODUCTION

Drosophila Wg belongs to a family of highly conserved proteins, the Wnt family, which have been implicated in both embryonic development and the regulation of cell proliferation in diverse species (reviewed by Nusse and Varmus, 1992; McMahon, 1992) . Wnt proteins are putative secreted glycoproteins that serve as signaling molecules in intercellular communication processes to control cell differentiation and proliferation. During *Drosophila* embryonic development, Wg plays a critical role in patterning the entire segmental unit to specify ventral naked cuticle and cell type diversity in the epidermis. In addition, Wg is also required for other developmental processes including segmental patterning of the midgut epithelium, development of Malpighian tubules, formation of the stomatogastric nervous system, specification of a subset of neuroblasts, and imaginal disc patterning (reviewed by Siegfried and Perrimon, 1994; Klingensmith and Nusse, 1994) .

Biochemically, Wnt family proteins possess a putative signal peptide, consensus sites for the attachment of N-linked oligosaccharides and 22 highly conserved cystein residues whose spacing is conserved. All the Wnt proteins examined to date enter the secretory pathway and are modified with N-linked oligosaccharides (Burrus and McMahon 1995) . Wnt proteins are poorly secreted into the cultured medium and levels of the proteins are increased by addition of the polyanions heparin or suramin (Burrus and McMahon 1995) , suggesting that the majority of secreted Wnt proteins are associated with the cell surface or the extracellular matrix (ECM). It has been demonstrated that both Wg and mouse Wnt-1 are tightly associated with the cell surface (Papkoff and Schryver 1990; van den Heuvel et al. 1993; Reichsman et al. 1996) and the ECM (Bradley and Brown 1990; Gonzalez et al. 1991; van den Heuvel et al. 1993; Reichsman et al. 1996) . The nature of this association is likely due to the interaction of the Wg/Wnt-1 proteins with heparan sulfate proteoglycans (HSPGs) since both of these proteins can be released by addition of exogenous heparin (Bradley and Brown 1990; Reichsman et al. 1996) . Further, heparin can directly bind to purified Wnt-1 (Bradley and Brown 1990) and Wg protein (Reichsman et al. 1996) . The association of Wg protein with the cell surface and the ECM has been shown to be important for its signaling activity since Wg signaling can be inhibited by removal of the heparan sulfate with heparinase or by treatment of cells with sodium perchlorate, a competitive inhibitor that blocks the sulfation of proteoglycans (Reichsman et al. 1996) . Thus, HSPGs can potentially play an important role in both the localization of Wg protein and its signaling activity during development.

While biochemical studies of Wnt signaling have been hampered by the difficulty of isolating active Wnt proteins, genetic studies in *Drosophila* have successfully identified some of the molecules involved in Wg signaling. Phenotypic and genetic interaction studies have identified four genes, *zeste white* 3 (*zw*3, also know as *shaggy*), *dishevelled* (*dsh*), *armadillo* (*arm*) and *porcupine* (*porc*) that act in Wg signaling (reviewed by Perrimon 1996) . The current model is that Porc is required for the secretion of Wg protein, whereas in the receiving cells, Wg initiates a signaling cascade through Dsh which inactivates Zw3 kinase. Inactivation of Zw3 results in the accumulation of cytoplasmic Arm protein which in turn regulates many downstream effectors of Wg, including the transcription factor *engrailed*

(*en*) in the embryonic epidermis and genes of the *achaete scute complex* at the wing margin (reviewed by Siegfried and Perrimon, 1994; Klingensmith and Nusse, 1994). Recently, compelling evidence has been obtained in tissue culture cells that *Drosophila Frizzled2* (*Dfz2*) functions as a Wg receptor in cultured cells (Bhanot et al. 1996) . Dfz2 is a member of the Frizzled family of proteins which possess a putative signal sequence, an extracellular domain composed of a conserved region of 120 amino acid residues with an invariant pattern of ten cysteines, seven putative transmembrane domains, and a short cytoplasmic domain (Wang et al. 1996) .

The role of *porc*, *zw3* and *dsh* in Wg signaling was originally recognized following examination of the segmentation phenotypes of embryos that lack both maternal and zygotic activities. These mutations were identified as the result of systematic screening of zygotic lethal mutations in germline mosaics (Perrimon et al. 1989) . I have recently identified a new segment polarity mutant which I named *sulfateless (sfl)* using this approach (Perrimon et al. 1996; and unpublished). In this annual report, I will describe the identification, characterization and the genetic studies of this mutant. I show that *sfl* mutations impair signaling by Wg, and that it encodes the homologues of vertebrate heparan sulfate (HS)/heparin N-deacetylase/N-sulfotransferase, an enzyme essential for the biosynthesis of HS/heparin proteoglycans (HSPGs). We find that Sfl acts non cell-autonoumously in Wg signaling and overexpression of Wg can bypass the requirement for this enzyme suggesting that HSPGs modulate Wg signaling, possibly by facilitating the binding of Wg to its receptor. These results are consistent with a model whereby HSPGs facilitate the interaction of Wg with its receptor Dfz2.

BODY

Materials and Methods

Genetics of sulfateless

A single *P[lacZ, ry$^+$]* element insertion, *l(3)03844* (Spradling et al. 1995) , located in 65C1-2 was identified in a screen for maternal effects of zygotic lethal mutations (Perrimon et al. 1996) . We found that *sfl* fails to complement *Df(3L)ZN47* that deletes the 64C to 65C region of the third chromosome. This result is in consistent with the location of a zygotic lethal mutation in the 65C1-2 region. Further, we mobilized the P-element associated with the *l(3)03844* line using the *y w; •2-3, Sb/TM6* strain (Robertson et al. 1988) . Out of 60 excision lines analyzed, 9 were viable in trans with *Df(3L)ZN47*, indicating that the P-element insertion at 65C is associated with zygotic lethality. These revertant lines fully rescued the maternal effect segment polarity phenotypes associated with *l(3)03844.*

Maternal effect phenotype of sfl

Females with germline clones were generated using the autosomal "FLP-DFS" technique (Chou and Perrimon 1996) . Briefly, virgin females of the genotype *sflFRT^{2A}/TM3, Sb* were mated with males of the genotype *y w FLP^{22}/+, FRT^{2A} P[ovo^{D1}]/TM3, Sb.* The resulting progeny were heat shocked at 37^{o}C for 2 hrs at the larval stages, and *y w FLP^{22}/+; sflFRT^{2A}/FRT^{2A} P[ovo^{D1}]* females carrying *sfl* homozygous germline clones were selected.

Antibody staining and in situ hybridization

Fixation of embryos and antibody staining procedures were performed as described (Patel 1994) . Anti-Wg serum was a gift of S. Cumberledge and used at 1:500 dilution. Anti-En MAb4D9 was used at 1:300 dilution and obtained from Developmental Studies Hybridoma Bank (Patel et al. 1989) . Antibody against the Crumbs protein was used at 1:50 and obtained from E. Knust (Tepass and Knust 1993) .

In situ hybridization of whole-mount embryos was done with PCR-generated digoxigenin-labeled DNA probe (Lehmann and Tautz 1994) . *wg* digoxigenin-labeled DNA probe was prepared from a*wg* cDNA subcloned in the p^{sp65} plasmid.

Molecular biology

Genomic DNAs flanking the *sfl* P-element insertions were obtained by plasmid rescue in *E. coli* (Cooley et al. 1988) . To isolate *sfl* cDNAs, we screened a 0-4 hr embryonic cDNA library (Brown and Kafatos 1988) with random primed probes generated from genomic DNA fragments from plasmid rescue. One full-length 4.89 kb *sfl* was isolated. DNAs were sequenced by Taq-polymerase cycle sequencing and an automatic sequencer. To define the P-element genomic insertion site, rescued plasmids from *sfl* flies were also sequenced using a primer derived from the P-element.

Northern blots of total RNA or polyA$^+$ RNA were carried out by standard procedures (Sambrook et al. 1989) . Probes used are as following: 2.5 kb Xho-EcoRI fragment of *sfl* cDNA (see Figure 2A for

sites), 1.87 kb SstI-XhoI fragment of the sugarless (UDP-D-glucose dehydrogenase). Sequence alignments were produced using "DNA star" software.

RNA injection rescue

To mark embryos for RNA injection rescues, the *sfl* mutations were recombined with a *trachealess* (*trh*) mutation (Wilk et al. 1996; Isaac and Andrew 1996) located at 61 C1-2. *trh* mutants are missing tracheas and exhibit defective posterior spiracles which are easily scorable by looking at embryonic cuticles. Females that carry *trh sfl* homozygous germline clones were generated and mated with *sfl/trh* transheterozygous males, respectively. Half of the embryos from this cross are *sfl* mutants and exhibit strong segment polarity phenotypes, while the remaining half of the embryos which are paternally rescued are easily identifiable by their defective posterior spiracles.

RNAs were produced by *in vitro* transcription (Sambrook et al. 1989) from full-length cDNA plasmids containing an SP6 promoter using cap analog GpppG (Stratagene). Transcribed RNA was resuspended in DEPC-treated water at concentration of 0.2µg/µl. Embryos generated from the cross described above were collected and microinjected as described (Anderson and Nüsslein-Volhard 1984) . Injected embryos were allowed to complete development for 3 days at 18^{o}C prior to preparation, and scoring of cuticles was as described (Wieschaus and Nüsslein-Volhard 1986) . Of 700 injected embryos derived from females with *trh sfl* germline clones, 120 *sfl* mutant embryos (*trh sfl/sfl*) developed scorable cuticle structures, and 15% of them were rescued

Misexpression experiments

The *pairedGAL4/TM3* (*prdGAL4*) line used in this study is described in Yoffe et al. (1995). *prdGAL4* was recombined with *sfl* FRT^{2A} chromosome(s) to generate *sfl prdGAL4* FRT^{2A} chromosomes that were used to generate homozygous germline clones. The $UASwg^{ts}$ (*M7-2.1*) line is located on the third chromsome and is described previously (Wilder and Perrimon 1995; Yoffe et al. 1995) . This insertion is homozygous viable. The $UASwg^{ts}$ was recombined with the *sfl* mutation to generate *sfl* $UASwg^{ts}$. *UAS Wg* was a gift of Henry Krause (unpublished). *UAS* Arm^{s10} was a gift of Mark Peifer (L.M, Pai, S. Orsulic, A. Bejsovec, and M. Peifer. submitted). The insertion we used is homozygous viable and located on the second chromsome. *UAShh* was obtained from. P. Ingham (Ingham and Fietz 1995)

Results

sulfateless (sfl) *is a novel segment polarity gene*

Mutations in *sfl* was originally identified in a large screen to characterize the maternal effects of zygotic lethal mutations (see Perrimon et al., 1996; and Materials and methods for details). Homozygous *sfl* mutant animals derived from heterozygous mothers die at the third instar larval or early pupal stages. In contrast homozygous *sfl* mutant embryos derived from females lacking germline *sfl* activity (referred to

as *sfl* null embryos throughout the text) die with segmentation defects that resemble the phenotypes of mutants in segment polarity genes (Figures 1B and 1C). The *sfl* maternal effect is complete paternally rescuable(see Perrimon et al., 1996; and Materials and methods for details) indicating that this gene is expressed in both oogenesis and early embryonic development.

A EMS allele of *sfl* has been generated through standard EMS mutagenesis. The nature of this EMS allele has not been determined, but it exhibit virtually identical defects examined. Since P1610 is a *sfl* RNA null mutant (See result later), we use this null mutant for all the other analyses.

The cuticle phenotypes of *sfl* is reminiscent of the phenotypes exhibited by either mutations in *wg* or *hedgehog (hh)* suggesting that they may be involved in either or both of these signaling pathways. To further determine the involvement of *sfl* in Wg or Hh signaling, I examined the expression of *wg* mRNA, Wg protein and En protein in *sfl* null mutant embryos. In the ventral embryonic epidermis, Wg signaling is required for maintenance of *en* transcription at stage 10 (DiNardo et al. 1988; Yoffe et al. 1995) . Subsequently, En, through a signaling pathway mediated by Hh (Ingham et al. 1991; Lee et al. 1992) , is required for the maintenance of *wg* transcription (Martinez-Arias et al. 1988; Bejsovec and Martinez-Arias 1991) . As shown in Figure 1, *wg* mRNAs fade in *sfl* (Figure 1D) mutant at stage 9. Similarly, *en* expression is affected because by stage 10, En proteins fade from the epidermis in *sfl* (Figure 1F)mutant embryos. Double stainings for both En and Wg protein indicate that when En proteins begin to fade during late stage 9 in *sfl* (Figure 1H) mutant, Wg protein has nearly completely decayed. These patterns of *wg* and *en* expression are reminiscent of those observed in either *wg* or *hh* null mutants (DiNardo et al. 1988; van den Heuvel et al. 1993; Yoffe et al. 1995; Manoukian et al. 1995; Alcedo et al. 1996) and are consistent with a role for *sfl* in either Wg and/or Hh signaling.

sulfateless *encodes heparan sulfate N-deacetylase/N-sulfotransferase*

To molecularly characterize the gene associated with the *sfl* mutation, I cloned the genomic DNA flanking the P-element insertion associated with *sfl* mutant following plasmid rescue. A DNA subfragment adjacent to the P-element insertion was used to probe a Northern blot and a 5.0 kb transcript was identified (data not shown). This DNA subfragment was then used to screen a 0-4 hr embryonic cDNA library. A 4.89 kb full-length cDNA clone was isolated and found to encode a conceptual protein of 1048 amino acid residues in length (Figure 2B).

Three lines of evidence suggest that the cDNA we isolated identifies *sfl*. First, the sequence of the cDNA, when compared to the genomic site of insertion of the P-element, revealed that the P-element is inserted 686 bp upstream of a putative ATG start codon in the untranslated 5' region of the cDNA (Figure 2A). This suggests that the P-element insertion may disrupt the *sfl* transcript. Second, Northern blots probed using a *sfl* cDNA detect no *sfl* maternal transcripts in 0-1.5 hr embryos derived from females with *sfl* germline clones, indicating that the P-element insertion disrupts the *sfl* transcript (Figure 3A). Third, *in vitro* transcribed *sfl* RNAs injected into marked *sfl* mutant embryos were able to rescue the embryonic null mutant phenotype (Figures 3B2 and 3B3).

A search of the protein sequence databases revealed that the putative protein deduced from *sfl* cDNA has striking homology with vertebrate heparan sulfate (HS) N-deacetylase/N-sulfotransferase (Hashimoto et al. 1992; Dixon et al. 1995) and heparin N-deacetylase/N-sulfotransferase (Orellana and Hirschberg 1994; Eriksson et al. 1994) , which are essential for the modification of HS and heparin polysaccharide chains respectively. As shown in Figure 2B, while the 160 N-terminal amino acid residues are distinct from either HS or heparin N-deacetylase/N-sulfotransferase, the remaining sequences are highly homologous. The overall identity between Sfl and rat HS N-deacetylase/N-sulfotransferase and mouse heparin N-deacetylase/N-sulfotransferase are 51% and 53% respectively. HS N-deacetylase/N-sulfotransferase and Heparin N-deacetylase/N-sulfotransferase are homologous enzymes with certain differences in function and expression. While HS N-deacetylase/N-sulfotransferase is ubiquitously expressed in all types of mammalian cells tested, the expression of heparin N-deacetylase/N-sulfotransferase is unique to connective tissue mass cells that produce heparin (Orellana and Hirschberg 1994; Eriksson et al. 1994) . These enzymes have been suggested to be derived from an ancestral gene (Eriksson et al. 1994) , Since *sfl* is expressed both maternally and ubiquitously throughout embryonic development (date not shown), Sfl may represent a prototype enzyme of vertebrate HS/heparin N-deacetylase/N-sulfotransferase.

The phenotype of sfl *in the stomatogastric nervous system*

The molecular nature of Sfl predicts that the biosynthesis of HS/heparin is affected in *sfl* mutant. Since HS/heparin has been demonstrated to be important in Wg signaling in tissue culture experiments (Reichsman et al. 1996) , it is anticipated that Wg signaling is affected in *sfl* and that the cuticle phenotypes of *sfl* mutant may reflect a role of HS/heparin in Wg signaling. However, studying the requirement for these genes in Wg signaling in the epidermis is complicated because of the codependence of Wg and Hh signaling (Ingham et al. 1991; Lee et al. 1992) . Thus, to determine the specificity of Sfl in Wg signaling, we examined the phenotype associated with *sfl* mutation in a tissue where the role of Hh and Wg is clearly distinguishable.

One of these tissues is the anlage of the stomatogastric nervous system (SNS) (González-Gaitán and Jäckle 1995) . The SNS arises during stage 10 from a distinctive region of the invaginating foregut. During stage 11 three invaginations form in the dorsal epithelium of the foregut (Figure 4A). Later these invaginations will form vesicles which migrate dorsally towards the brain to form the SNS. In *wg* mutant embryos only one of three invaginations forms (Figure 4B). The large size of this invagination suggests that initially the same number of cells are determined to form the SNS but then fuse into one. Virtually identical phenotypes, namely only one oversized invagination, are observed in embryos mutant for genes previously shown to be involved in Wg signaling like *dsh* and *arm* (González-Gaitán and Jäckle 1995) . In contrast, in *zw3* mutant embryos, additional invaginations are observed, which is consistent with the opposite phenotypes of *wg* and *zw3* mutants in the ventral cuticle (Siegfried et al. 1992) . Altogether, the Wg signaling pathway in the ventral cuticle and in the development of the SNS appears identical (González-Gaitán and Jäckle 1995) . In contrast to *wg* mutants, *hh* mutants, like wildtype embryos form

three SNS invaginations. Although *hh* is expressed in the SNS Anlage we could not detect any defects specifically associated with the SNS in *hh* mutants (data not shown).

In order to examine the role of *sfl* in the development of the SNS anlage, *sfl* null mutant embryos were stained with an antibody against the Crumbs protein that labels the apical surfaces of epithelial cells throughout the embryo (Tepass and Knust 1993) . In contrast to the phenotypes seen in genes previously implicated in transducing the Wg signal, we did not observe the one-large-invagination phenotype in *sfl* embryo. Instead in *sfl* mutant embryos, 2-3 invaginations that are fused at the base are usually detected (figures 4C). This phenotype is reminiscent of a weak *wg* mutant phenotype, as observed in a weak *arm* allele (see Figure 8I in González-Gaitán and Jäckle, 1995). However, in contrast to the ventral epidermis where the phenotype of *sfl* resemble a *wg* null phenotype the phenotype in the SNS is less severe, suggesting that Wg signaling in the SNS is not completely abolished in these mutants but that residual Wg signaling occurs. Interestingly, in contrast to the ventral epidermis of *sfl* mutant embryos, where Wg expresssion is lost, Wg expression persists in the SNS anlage (Figure 4E. also see figures 1D, 1F, 1H). This observation suggests that the difference in severity of phenotypes between the SNS and the ventral epidermis may originate from the different modes of Wg regulation deployed.

We have also examined the phenotypes of malpighian tubules which require Wg activity for their development (Skaer and Martinez arias, 1992) As we observed for the SNS, the malpighian tubules of *sfl* mutant embryos are partially defected (data not shown) and these phenotypes are reminiscent of a weak *wg* mutant phenotype. These observations further support the model that Wg activity is reduced but not completely abolished in some tissues of *sfl* mutant.

Ectopic expression of Wg can bypass the requirement for Sfl

Studies in the SNS and the malpighian tubules suggest that Sfl is not absolutely essential in all tissues to implement the Wg signal, but instead may be involved in modulating the strength of the cellular response to the Wg signal. To test this hypothesis we examined the effect of ectopic expression of Wg in the ventral epidermis of *sfl* null mutant embryos. We reasoned that if Sfl promote the cellular response to Wg signal, ectopic expression of various amounts of Wg should elicit dosage dependent effects. We used the GAL4/UAS system of targeted gene misexpression (Brand and Perrimon 1993) to misexpress various forms of Wg in *sfl* mutant embryos. A prdGAL4 line was used that drives the expression of a UAS-target gene in the *paired* (*prd*) pair rule expression domain from stages 8 to 13 (Yoffe et al. 1995) .

We first examined whether ectopic expression of Wg^{ts} effectively restores the naked cuticle as it does in *wg* and *porc* mutant embryos (Yoffe et al. 1995; Manoukian et al. 1995) . In wild type embryos, ectopic expression of Wg is associated with ectopic *en* as well as generation of naked cuticle (Yoffe et al. 1995) . As shown in Figure 5, in *sfl* mutant embryo, expression of *prdGAL4/UAS*wg^{ts} does not generate naked cuticles (Figures 5A), while in control experiments, *prdGAL4/UAS*wg^{ts} induced the expansion of En stripes in either *sfl* or *sgl* zygotic mutant embryos with maternal supply (data not shown) as was shown previously (Yoffe et al. 1995) . This result demonstrates that *sfl* and *sgl* are required for implementation of the Wg signal.

In light of the requirement for these enzymes in Wg signaling during SNS formation, we reasoned that the inability of Wg^{ts} to generate naked cuticle in *sfl* mutant embryo may be due to the low activity of Wg^{ts} under these experimental conditions. These experiments were conducted at 16°C, which is permissive for Wg^{ts} (van den Heuvel et al. 1993; Wilder and Perrimon 1995) . At this temperature, GAL4 is not as efficient as at higher temperature (Brand et al. 1994) , and the activity of Wg^{ts} may be relatively low. Thus, we repeated the same misexpression experiment in the embryonic epidermis using a *UAS-wild type wg* construct. In wild type embryos, expression of wild type Wg protein in the paired expression domain results in deletion of denticle bands when embryos are allowed to develop at either 25°C (Figure 5D) or 16°C (Figure 5C). Wild type Wg protein has stronger activity than Wg^{ts} since expression of Wg^{ts} in the identical condition only causes the deletion of one row of denticles (Yoffe et al. 1995). Strikingly, at 25°C, expression of *prdGAL4/UASwg* in *sfl* mutant embryo induces naked cuticle (Figures 5D) demonstrating that Wg signaling can occur in the absence of these enzymes. However, we observed only a weak effect of ectopic Wg, indicated by the presence of narrow naked cuticles when the same experiment was conducted at 16°C (Figures 5E). These results indicate that ectopic expression of Wg can rescue the defects of cuticle patterning in *sfl* mutant in a dose dependent manner.

We also examined the effect of misexpression of Hh in the paired domain in *sfl* null mutant embryos. As shown in Figures 5. *prdGAL4/UAShh* can effectively induce naked cuticles in *sfl* (Figure 5G) null mutant embryos. Strikingly, at stage 11 embryos in *sfl* mutant (Figures 5H), Wg protein is maintained/induced in the paired domain where ectopic Hh is expressed. This result is consistent with the Wg misexpression experiments because the effect of Hh is mediated by *wg*.

To further determine at what level Sfl and Sgl are required in Wg signaling, we examined the epistatic relationships between Sfl and Arm by misexpressing a gain of function Arm protein (Arm^{S10}) in which 54 amino acid residues in N-terminal of Arm are deleted (L.M, Pai, S. Orsulic, A. Bejsovec, and M. Peifer. submitted). As shown in Figures 5F, expression of this constitutively active, Wg-independent form of Arm under the control of *prdGAL4*, in *sfl* null embryos lead to the formation of naked cuticle in the paired domain. This result indicates that Arm is epistatic to Sfl. The formation of naked cuticle is most likely the result of ectopic expression of *wg*, triggered by ectopic *en* and *hh* that occur in response to ectopic expression of Arm^{S10} (Pai et al. 1997) .

Sfl is required for Wg signaling in wing imaginal Disc and acts cell nonautonomously

Our results suggest that Sfl is required for wg signaling, possibly by modulating the strength of Wg activity during embryonic development. Since Wg activity is also required for development of imaginal discs, such as the specification of wing margin structure, we therefore would like to examine if Sfl is also required for Wg signaling in imaginal discs. As shown in Figure 6B and C, clones of *sfl* in wing margin phenocopy clones of wg and dsh mutation, suggesting that Sfl is required for Wg signaling in the development of wing imaginal discs. To test the autonomy of *sfl* function, a yellow mutation was used to

mark sfl clones. Making yellow marked clones of sfl demostrated that Sfl functions non cell-autonomously(Figure 6D and 6E).

CONCLUSIONS

Sfl is *involved in* Wg *signaling in both embryonic and imaginal discs development*

I have identified and characterized a novel segment polarity gene, *sfl*, which encodes the homologue of vertebrate heparan/haparin sulfate N-deacetylase/N-sulfotransferase. Embryos that develop in the absence of both maternal and zygotic *sfl* gene product exhibit cuticle defects and defects in gene expression in the ventral epidermis identical to those observed in *wg* or *hh* mutant embryos. To demonstrate that normal expression of these genes are required for proper Wg signaling, I analyzed the requirement for these genes in SNS formation where *wg* expression does not depend on Hh activity. Interestingly, I found that Wg signaling is only partially blocked in *sfl* null mutant embryos, during SNS formation. Partially defects of Wg signaling are also observed in the formation of malpighian tubule of *sfl* mutant embryos. These observations suggest that implementation of the Wg signal can occur in some tissues in the total absence of these gene activities. In support of this hypothesis, misexpression of wild type Wg protein, but not of a less effective Wg protein, can rescue the naked cuticle in *sfl* embryo.
Our results also demonstrate that Sfl is required for Wg signaling in wing imaginal discs. Clones of *sfl* disrupt wing margin structures, phenotypes reminiscent to the defects of clones of wg or dsh. Sfl acts non cell-autonomously in determining wing margin structure suggesting that a HSPG(s) involved in Wg signaling can move from cell to cell. It has been demonstrated that glypican family HSPG can move from cell to cell possibly via a GPI anchor, while syndecan family HSPG can be shed from cell surface.

Our result is consistent with an view that a glypican or a syndecan- like HSPG is likely involved in Wg signaling. Experiments to address this issue has been carried out currently. Further genetic and biochemical studies should reveal whether a Glypican and/or Syndecan play a direct role in Wg signaling.

sfl *encods enzyme involved in HSPGs biosynthesis*

HSPGs are ubiquitous macromolecules associated with the cell surface and the ECM of a wide range of cells that play central roles in embryonic morphogenesis, neurite outgrowth, angiogenesis, and tissue repairs (reviewed by Bernfield et al., 1992; David, 1993; Kjellén and Lindahl, 1991; Yanagishita and Hascall, 1992). The biological function of HSPG are mostly attributed to the highly negatively charged Glycosaminoglycans (GAGs) chains that interact with positively charged side chain of proteins.
Sfl protein is strikingly homologous with vertebrate HS N-deacetylase/N-sulfotransferase and heparin N-deacetylase/N-sulfotransferase. These proteins are homologous enzymes with certain differences in expression and function (Hashimoto et al. 1992; Eriksson et al. 1994; Orellana and Hirschberg 1994; Dixon et al. 1995) . While HS N-deacetylase/N-sulfotransferase is ubiquitously expressed in all mammalian cells examined, the expression of heparin N-deacetylase/N-sulfotransferase is unique to heparin-producing cells. These enzymes have been suggested to be derived from an ancestral gene

(Eriksson et al. 1994) . Since *fl* is expressed both maternally and ubiquitously throughout embryonic development, it is likely that Sfl represents a homologue of HS N-deacetylase/N-sulfotransferase or a prototype enzyme of vertebrate HS/heparin N-deacetylase/N-sulfotransferase. In vertebrates, HS is a ubiquitous GAG that is closely related to heparin, a GAG expressed *in vivo* solely as a proteoglycan within the granules of mast cells and basophils (reviewed by Bernfield et al., 1992; Kjellén and Lindahl, 1991) . Biosynthesis of HS and heparin are similar in vertebrates and invertebrates, and are initiated by formation of a polysaccharide chain consisting of (D-glucuronic acid••••N-acetyl-D-glucosamine••••$_n$••Subsequently, several modifications including N-deacetylation and N-sulfation, Uronosyl C5-epimerization, 2-0-sulfation, and 6-0-sulfation occur (reviewed by Kjellén and Lindahl, 1991) . HS/heparin N-deacetylase/N-sulfotransferase catalyzes N-deacetylation and N-sulfation that is the first and key step to initiate further modification reactions of HS/heparin. Since sulfation and epimerization of HS/heparin molecules provide structural identity and negative charges which are critical for its interaction with proteins, loss of Sfl protein will result in the generation of unmodified HS/heparin and thus impair the normal function of HSPGs.

In conclusion, biochemical evidence strongly argues that biosynthesis of HSPGs rather than other proteoglycans is specifically affected in *sfl*. Since *sfl* mutant embryos exhibit defects in Wg signaling in both embryonic and imaginal disc development, we conclude HSPGs rather than other proteoglycans are required for Wg signaling. Thus, we propose that in the absence of Sfl protein, Wg signaling is perturbed due to absence of modification. If the general synthesis of HSPGs is perturbed in *sfl* mutant, then it is expected that other signaling pathways, which utilize HSPGs as coreceptor(s), may also be disrupted in the absence of Sfl and Sgl proteins. Consistent with this hypothesis, we have observed that mesoderm migration in *sfl* mutant is defective (X. Lin, A. Michelson and N. Perrimon, unpublished) as seen in the embryos with mutations of *heartless* , a *Drosophila* homologue of FGF receptor (Beiman et al. 1996; Gisselbrecht et al. 1996) . The availability of either *sfl* mutant will allow us to examine the roles of HSPGs in signaling mediated by other growth factors including FGFs.

A model of the role of HSPGs in Wg signaling

Among the growth factors that interact with proteoglycans, members of the FGF family have been particularly well studied. It has been proposed that HSPGs are required for dimerization of FGF receptors (Spivak-Kroizmon et al. 1994; Schlessinger et al. 1995) . In an alternative model, HSPGs have been proposed to reduce the dimensionality of ligand diffusion from three to two dimensions (Schlessinger et al. 1995) . Our genetic experiments demonstrate that in *sfl* mutant embryos, overexpression of Wg protein can bypass the requirement of HSPGs to transduce Wg signal to receiving cells. Based on these results, we propose that the function of HSPGs is to increase the local concentration of Wg ligand for its receptor (Figure 7). Binding of Wg protein to HSPGs on the cell surface or the ECM reduces the diffusion of the Wg ligand so that the Wg molecules are more likely to bind to the less abundant Wg receptor. In the absence of HSPGs, the concentration of Wg protein presented on the cell surface may be lower than its threshold concentration, and the efficiency of Wg

signaling will be reduced. Overexpression of ectopic Wg protein can compensate the loss of the Wg protein on the cell surface and therefore bypass the requirement of HSPGs to transduce Wg signal to receiving cells.

This model is consistent with our observation in the SNS and in the malpighian tubules where Wg signaling is not completely abolished in either *sfl* or *sgl* mutants. In the ventral epidermis however, the situation appears different as the loss of Sfl and Sgl activities mimic the complete loss of Wg activity. This observation most likely reflects the fact that in the ventral epidermis, the maintenance of *wg* transcription requires Wg signaling itself, either directly in an autocrine pathway, or via the Hh feed back loop. In the epidermis, an initial reduction in the efficiency in Wg signaling may rapidly lead to a loss of *wg* transcription and thus resemble a complete loss of Wg activity.

Growth factors and HSPGs

Accumulating evidence has demonstrated that coreceptors, such as HSPG, function as indispensable components for signaling of a number of growth factors (Schlessinger et al. 1995; Massagué 1996) . For example, FGFs require HSPGs as a coreceptor for signaling via a tyrosine kinase receptor. TGF-••binds to serine/threonine kinase receptor in association with the membrane protein betaglycan (Schlessinger et al. 1995; Massagué 1996) . Either GDNF or CNTF require GPI-anchored protein to mediate its signaling (Massagué 1996; Stahl and Yancopoulos 1993) . Binding of growth factors to coreceptors, such as cell surface HSPGs will not transmit signals alone, but modulate the ability of growth factors, or the signaling receptors, to generate a biological response.

While the function of these molecules has been proposed to limit ligand diffusion from the cell surface, and to initiate dimerization of receptors, we speculate that these molecules may mediate other functional aspects of growth factors. For instance, Wg protein functions both as a short range inducer in the ventral epidermis (van den Heuvel et al. 1989; DiNardo et al. 1988; Vincent and Lawrence 1994) and as a long-range organizer in imaginal discs (Struhl and Basler 1993; Diaz-Benjumea and Cohen 1995) . Recent evidence suggests that Wg can act directly and at long range as a gradient morphogen (Zecca et al. 1996) . If HSPG is a major limiting factor for diffusion of Wg protein, one would expect that dynamic changes of the expression of HSPGs may regulate the diffusion of Wg and eventually control the signaling range of Wg protein. Interestingly, a Glypican-related HSPG, named Dally, was identified, and *dally* mutants show wing notching with loss of wing margin structures (Nakato et al. 1995) , an effect seen in the either *wg* and *dsh* mutants (Couso et al. 1994) , suggesting a potential involvement of Dally in Wg signaling. A *Drosophila* homologue of vertebrate Syndecans has also been characterized (Spring et al. 1994) . Syndecan is a transmembrance HSPG and represents the major source of HSPG in epithelial cells. Syndecan has been demonstrated to function as a coreceptor for FGF signaling (Bernfield et al. 1992; David 1993) . Further genetic and biochemical studies should reveal whether Dally and/or Syndecan play a direct role in Wg signaling.

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Figure Legend

Figure 1. Segment polarity phenotypes associated with *sfl* mutation.
The cuticle phenotypes of wild-type (A), *sfl* (B) embryos are shown; Transcription of *wg* mRNA is shown in stage 9 wild-type (C), and *sfl* (D) embryos; The expression of En protein is shown in stage 10 wild-type (E), *sfl* (F) embryos. The expression pattern of both Wg (blue/black) and En protein (brown) is shown in stage 10 wild type (G), late stage 9 *sfl*(H) embryos.

Figure 2. Molecular characterization of *sfl*.
(A) A restriction map of the *sfl* cDNA. The map is shown for the following restriction enzymes: B. BamHI, E. EcoRI, H. Hind III, S. SstI, X. XhoI. The insertion of the P-element *l(3)03844* is indicated by the arrow and corresponds to base 576 of the *sfl* cDNA (686 bp upstream of a putative ATG start codon). The position of the open reading frame encoding Sfl protein is indicated by the heavy line.
(B) Putative amino acid sequence of Sfl protein (I) and comparison of the predicted Sfl protein (I) to Rat heparan sulfate N-deacetylase/N-sulfotransferase (II) and Mouse Heparin N-deacetylase/N-sulfotransferase (III). Identical residues are enclosed in a box.

Figure 3. Rescue of *sfl* maternal effect phenotype by RNA injection.
A. Northern blot analysis of *sfl* RNA from 0-1.5 hr wildtype embryos and embryos derived from females with *sfl* germline clones. Equal amounts of RNA from 0-1.5 hr wildtype embryos were used as controls. About 60 •g total RNA were loaded. The blot was probed with ^{32}P labeled 2.5 kb Xho-EcoRI fragment of *sfl* cDNA (see Figure 2A for sites). A 1.87 kb SstI-XhoI fragment of sugarless (a Drosophila homologue of UDP-D-glucose dehydronase *l* cDNA was used as an internal control. In blot A, 5.0 kb and 2.3 kb mRNAs correspond to *sfl* and sugarless transcripts respectively.
B. B1. Cuticle phenotype of a paternally rescued *sfl* embryo marked with a *trh* mutation (see Materials and methods for details). Note the defective posterior spiracles. (B2) and (B3) are two examples of the cuticle phenotypes of *sfl* null embryos derived from germline clones which have been injected with RNA transcribed from the 4.89 kb *sfl* cDNA. Note that (B2) is fully rescued embryo in which the ventral denticle bands, head and tail structures are fully restored; (B3) is a partially rescued embryo.

Figure 4. Development of the stomatogastric nervous system (SNS) in *sfl* mutant.
The SNS stained by anti-Crumbs antibody in wild type (A), *wg* (B), *sfl* (C) is shown. The SNS is derived from three invaginations forming in the dorsal epithelium of the developing foregut of wildtype embryos at stage 10 (A). In a *wg* mutant embryo only one large invagination is formed (B). In *sfl* (C) mutant embryo three invaginations are formed and fused at the base. This phenotype is reminiscent of *wg* hypomorphs and suggests that in *sfl* mutant Wg activity is reduced but not completely abolished.

The expression of Wg protein in stage 10 embryos of wild type (D) and *sfl* (E) is shown. In contrast to *wg* expression in the ventral epidermis, which is lost (see Figures 1D, 1F, 1H), *wg* expression in the SNS anlage is retained in *sfl* (E) mutant.

Figure 5. Misexpression of wg^{ts}, *wg*, *hh* and arm^{s10} in *sfl* mutant.

Except for (B) and (C) which are controls of *UASwg/prdGAL4* in wild type backgrounds, all other panels show embryos derived from females with *sfl* germline clones. The cuticle phenotypes shown are: *sfl prdGAL4/sfl* $UASwg^{ts}$ (A), *UASwg/+; prdGAL4/+* (B and C), *UASwg/+; sfl prdGAL4/sfl* (D and E), *UAShh/+; sfl prdGAL4/sfl* (F), $UASarm^{s10}$*/+; sfl prdGAL4/sfl* (G). Embryos shown in (C) and (E) developed at 16°C. All the others developed at 25°C.

Wg protein expression is shown in a stage 11 embryo of *UAShh/+; sfl prdGAL4/sfl* (H). Note in (H) that while the stripes of endogenous Wg protein fade completely, new stripes of Wg proteins are strongly induced by ectopic expression of Hh.

Figure 6. Sfl is required for Wg signaling in wing imaginal discs and acts cell nonautonomously.

A. A wild type wing with intact wing margin structure,

B and C. clones of sfl mutation in wing margin disrupt wing margin structure and lead to deletion of wing margin indicated as arrows.

D. and paths of sfl mutant cells marked by y marker maintain the structure of wingmargin. The sfl mutant cells marked by y marker are indicated by arrow.

Figure 7. Model of the role of HSPGs in Wg signaling.

Secreted Wg protein binds heparan sulfate proteoglycan via interaction with heparan sulfate chains. HSPGs reduce the diffusion of Wg and increase the efficiency of ligand/receptor interaction (see text for discussion)

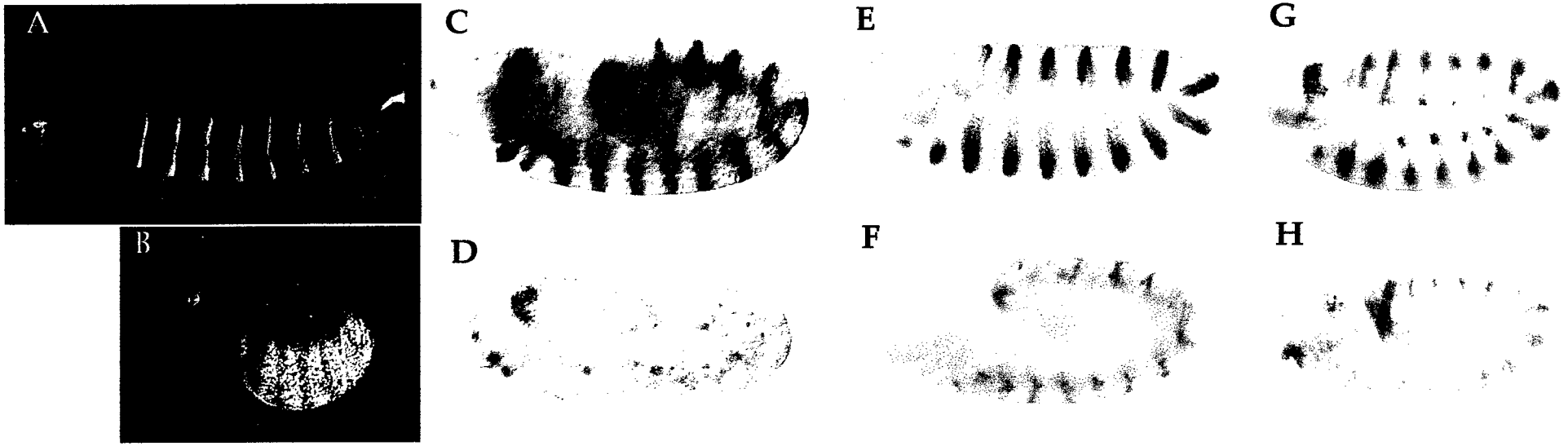
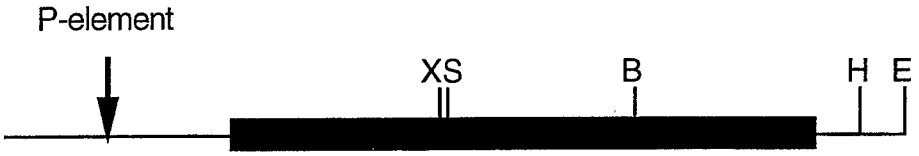


FIG. 1

A



B

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1    MTISGGNQHNNNANRKYEKLIKQPQMQFGSSVTGTQTDVDSCRDADADANAVRQDFSNFNKHFGNGHAITDRTMLLRLEDDVTTAAGIVT I
1    MP---------------------------------------------------------------------------------------- II
1    ML---------------------------------------------------------------------------------------- III

91   YKGKSNGNGNGNGNGSIGSISLDFNGSPTSSTSIGIASGSSSNTHLASGGGVGGIGGSEPAGWMCHCCNLIARRCFGINVRRCVLAILAI I
3    ----------------------------------------------ALAC---------------------LRRLCRHLSPQAVLFLLFVF II
3    ----------------------------------------------QLWK---------------------VVSPARQLELHRLILLLIGF III

181  TMVSIFYYTHYVDTGVFNGLIQRDTHPAPII---NCRMINSGGK-HIRNASPAPDHRSEARLRIDFKVLVFVETTYSGLGRDIAELLVYN I
27   CLFSVEVSAYYLYGW------NRGLEPSADASESDC---GDPPPVAPSRLLPIKPVQAVAPSRTDFLVLVFVESLYSQLGQEVVAILESS II
27   SLVSMGFLAYYVSTS------PKAKEPLP-LPLGDCSSSGAAGPGPARPPVPPREQRPPETTRTEEVVLVFVESAYSQLGQEIVAILESS III

267  RLKYKIEVA-GKS-LPVLTNLDKGRYGVIVFENLDKYLNMDKVWNRELLDKYCREYSVGIVGFVSPSEETLVGAQLRDFPLFVNTNLRLRD I
108  RFKYRTEIAPGKGDMPTLTDKGRGRFALIIYENILKYVNLDAWNRELLDKYCVAYGVGIIGFFKANENSLLSAQLKGFPLFLHSNLGLKD II
110  RFRYSTELAPGRGDMPTLTDHTHGRYVLVLYENLLKYVNLDAWSRELLDRYCVEYGVGIIGFFRAREHSLLSAQLKGFPLFLHSNLGLRD III

355  ASLNPLSSVIRLTRAGETAWGALPGDDWAVFQHNHSTYEPVEWAQRNTQEYPADSVGQVQL----PLTTVLQDRGQLDGIQRVLFGSSLR I
198  CSINPKSPLLVVTRPSEVEKGVLPGEDWTVFQSNHSTYEPVLLAKTRSSE-SIPHLGADAGLHAALHATVVQDLGLHDGIQRVLFGNNLN II
200  YQVNPSAPLLHLTRPSRLEPGPLPGDDWTIFQSNHSTYEPVLIASHRPAELSMP----GPVLRRARLPTVVQDLGLHDGIQRVLFGHGLS III

441  FWLHRLVFLLDALSVLSHGQLSLNLERMILVDIDDIFVGEKGTRLRPDDVRALIATQKNIAAMVPGFRFNLGFSGKYVYHHGTREENLGDDF I
287  FWLHKLVFVDAVAFLTGKRLSLPLDRYILVDIDDIFVGKEGTRMKVEDVKALFDTQNELRTHIPNFTFNLGYSGKFFHTGTDAEDAGDDL II
286  FWLHKLVFVDAVAYLTGKRLCLDLDRYILVDIDDIFVGKEGTRMKVADVEALLTTQNKLRTLVPNFTFNLGFSGKFYHTGTEEEDAGDDM III

531  LLQNVQEFNWFSHMWKHQQPHLVDNLTLLMAEMHLNYAFAVDHNIPTDSGYSISPHHSGVYPAHELLYMAWKKVWNVKVTSTEEYPHLRP I
377  LLSYVKEFWWFPHMWSHMQPHLFHNQSVLAEQMALNKKFAVEHGIPTDMGYAVAPHHSGVYPVHVQLYEAWKQVWNIRVTSTEEYPHLKP II
376  LLKHRREFWWFPHMWSHMQPHLFHNRSVLADQMRLNKQFALEHGIPTDLGYAVAPHHSGVYPIHSQLYEAWKSVWGIQVTSTEEYPHLRP III

621  ARLRRGFIHRNIMVLPRQTCGLFTHTMYIDRYPGGRDKLDESIQGGELFQFIVYNPINIFMTHMSNYGSDRLALYTFQSVIKFLQCWTNL I
467  ARYRRGFIHNGIMVLPRQTCGLFTHTIFYNEYPGGSSELDKIINGGELFLTVLLNPISVFMTHLSNYGNDRLGLYTFKHLVRFLHSWTNL II
466  ARYRRGFIHNGIMVLPRQTCGLFTHTIFYNEYPGGSRELDRSIRGGELFLTVLLNPISVFMTHLSNYGNDRLGLYTFESLVRFLQCWTRL III

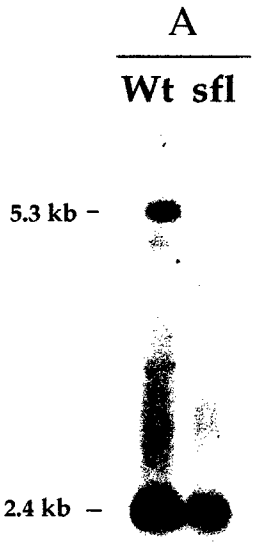
711  KLASAPPVQLAEMYFRLHPEEVDPVWGNPCDDVRHKKIWSKTKNCLSLPKFLVIGPQKTGTTALYTFLSMHGSIASNIASPETFEEVQFF I
557  RLQTLPPVQLAQKYFQIFSEEKDPLWQDPCEDKRHKDIWSKEKTCDRFPKFLIIGPQKTGTTALYLFLGMHPDLSSNYPSSETFEEIQFF II
556  RLQTLPPVPLAQKYFELFPQERSPLWQNPCDDKRHKDIWSKEKTCDRLPKFLIVGPQKTGTTAIHFFLSLHPAVTSSFPSESTFEEIQFF III

801  NGNNYYRGLDWYMDFFPSESLPNTSSPMPTQLGSPRFMFEKSATYFDGPAVPKRSHALLPHAKIVTILISPAKRAYSWYQHQRSHGDVLA I
647  NGHNYHKGIDWYMEFFP----------IPSN-TTSDFYFEKSANYFDSEVAPRRAAALLPKAKVLTILINPADRAYSWYQHQRAHLDFVA II
646  NGPNYHKGIDWYMDFFP----------VPSN-ASTDFLFEKSATYFDSEVVPRRGAALLPRAKIITVLINPADRAYSWYQHQRAHGDPIA III

891  NNYSFYQVITASDSAFRALKDLRNRCLNPGKYAQHLEHWLAYYPAQQLHIIDGEQLRLNPIDVMNELQRFLKIQPLLDYSNHLRYDVKKG I
726  LKYTFHEVITAGPDASSKLRALQNRCLVPGWYATHIERWLSAFHANQILVLDGKLLRTEPAKVMDTVQKFLGVTSTVDYHKTLAFDPKKG II
725  LNYTFYQVISASSQAFLLLRSLQNRCLVPGYYSTHLQRWLTYYPSGQLLIMDGQELRVNPAASMEIIQKFLGITPFLNYTRTLRFDEDKG III

981  FYCQAVSEKRNKCLGKSKGRQYPAMDERSAKLLQRYYLNHNTALVKLLKKLGSRPIPQWLKDDL-STGT.                    I
816  FWCQLLEGGKTKCLGKSKGRKYPEMDLDSRAFLKDYYRDHNIELSKLLYKMG-QTLPTWLREDLQNTR                      II
815  FWCQGLEGGKTRCLGRSKGRRYPDMDMESRLFLTDFFRNHNLELSKLLSRLG-QPAPLWLREELQHSSVG                     III
```

Lin, et. al., Figure 2



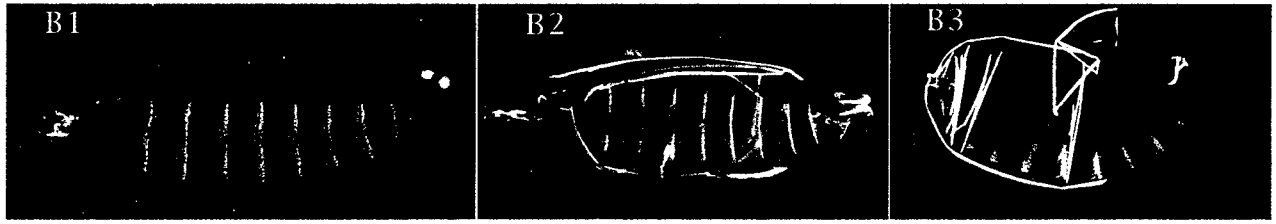


FIG 3

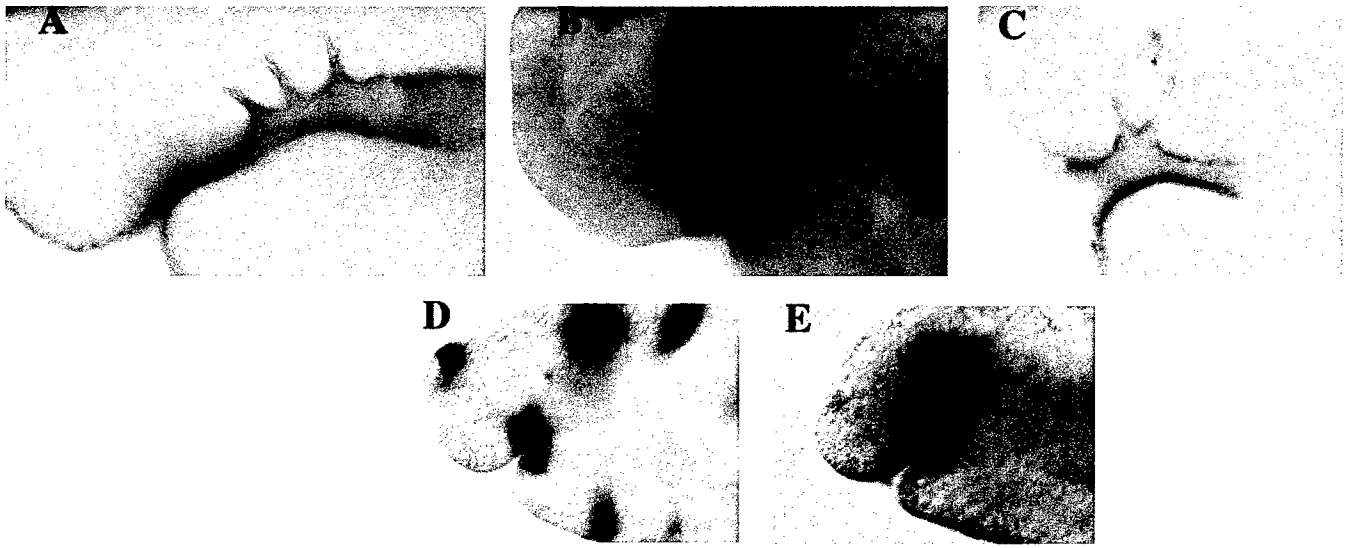
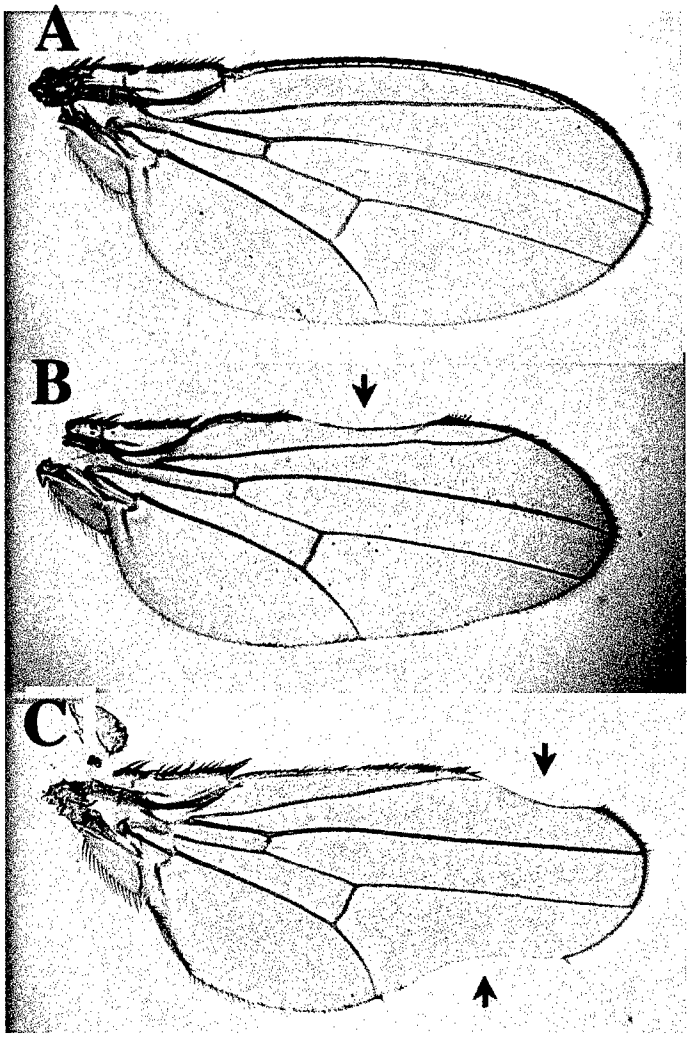


FIG 4

FIG 5



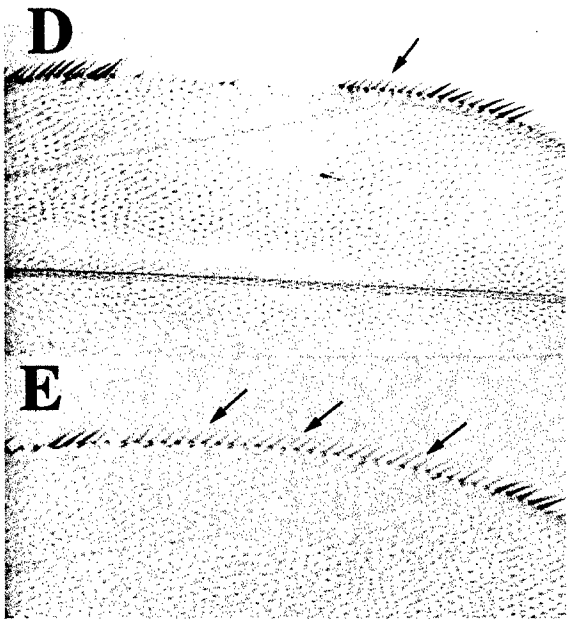
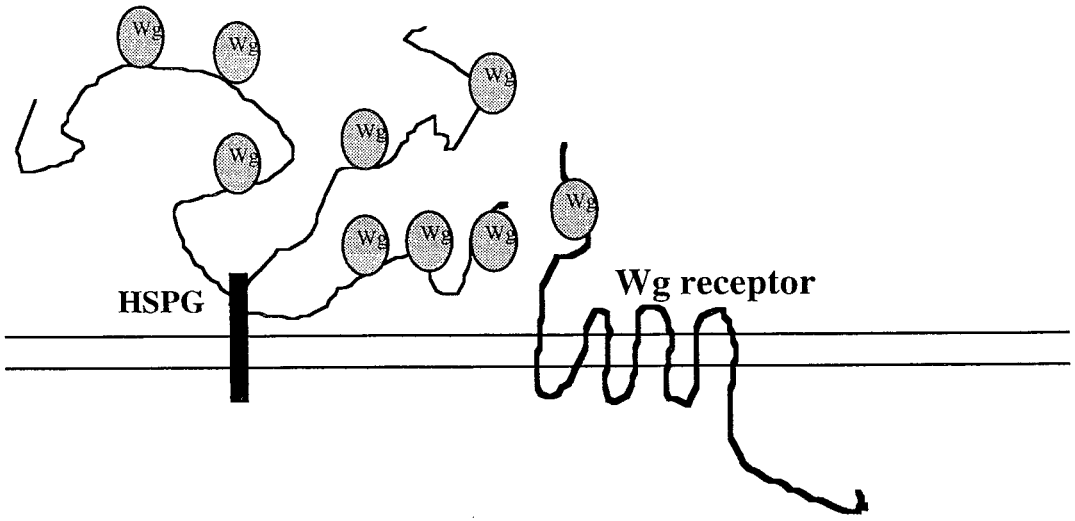


FIG 6



Lin, et. al., Figure 7

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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